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Specification, as originally filed, with Application for Patent Serial No: **2,286,414**, on October 4, 1999, by **ZHIBO GAN**, for "Non-Separation Heterogenous Assay for Biological Substance".

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Non-separation heterogenous assay for biological substances

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ABSTRACT OF THE DISCLOSURE

This present invention is for a method referred to as non-separation heterogenous assay that greatly simplifies the detection, identification , measurement of concentration and activity of biological substances. It is based on the change of the label signal due to the uneven distribution of the label between surface and liquid in a vessel after completion of the reaction among reactants. The method involves the coating of a reactant (labeled or unlabeled) onto a surface, addition of a sample with or without a competitor labeled using a label tag or unlabeled. The change of the label signal can be directly measured.

BACKGROUND OF THE INVENTION

Binding assays comprise a variety of methods that utilize the specific reaction between a ligand and corresponding binding agent such as protein. Immunoassay, receptor binding assays and lectin binding assay are examples of the binding assays. Immunoassay is by far the most common form which has been developed in to an extremely versatile analytic technique with a diverse range of assay protocols.

The first quantitative precipitation test and purified antibody for the first time was developed by Heidelberger (1939). The practicability of this principle received wide attention and led to its exploitation in simple immunoassays that could measure single antigen systems. The diffusion of antibodies and antigen in agar gel (Oudin, 1946), immuno-electrophoresis (Grabar and Williams, 1953), antiglobulin test (Coombs, 1945), fluorescent labeled antibodies (Coons, 1941) etc has been used for the assay. A milestone in sensitive assays was radioisotopic labeling techniques for antibodies and antigens (Farr, 1958). The efforts formed the basis of the radioimmunoassay (RIA) which has been rapidly adapted by researchers and clinical laboratories. Nakane and Pierce (1966) demonstrated that enzyme could be coupled to antibody or antigen. The importance of this discovery is reflected in the now widespread application of chromogenic, fluorogenic, luminescent signals for the measurements with a similar sensitivity to that of RIA. These discoveries led to the development of enzyme linked immunosorbent assay (ELISA) by Engvall and Perlmann (1971) which is widely used for researches and clinical tests at present.

Both RIA and ELISA involve the coating and separation of labeled and unlabeled antigen or antibody after the binding. These procedures inevitably use heterogenous phase and require

separation of antigen and/or antibody unbound to the surface, this type of immunoassay, therefore, is called "heterogenous" immunoassay. Rubenstein et al (1972) developed a new immunoassay using an enzyme as a label in which the antigen-antibody reaction and its measurement are performed in solution without the need of prior separation of the free and antibody-bound components and without the use of solid phase. This type of separation-free immunoassay is called "homogenous" immunoassay.

Most enzyme assays are carried out for the purpose of estimating the amount or activity of an enzyme present in a cell, tissue, other preparation, or as an essential part of an investigation involving the purification of an enzyme. The current assay methods have been developed based on the physical, chemical and immunological properties where they can be detected using photometric, radiometric, high performance liquid chromatographic, electrochemical assays, etc. (Eisenthal, R. and Danson, M. J., 1993). Although the methods basically fulfill the many essential requirements for routine analysis, there are, among those, the varying disadvantages of low sensitivity (Brenda Oppert et al, 1997), multiple steps (Twining, S. S., 1994) and steps that are tedious and time-consuming (Fields, R., 1976). Immunoassays have been widely using in human clinical tests and therapeutics, agriculture, food, veterinary and environmental diagnostics. In the most cases, immunoassays are effective and valid (Cleaveland, J. S. et al 1990), but in some cases they are not suitable, for example, in the determination of enzyme activity. This occurs because the binding assays for antibody and antigen (enzyme) can only be used to measure the concentration of an antigen (enzyme) but not its activity. It is of important to know the catalytic activity of an enzyme and not just the amount of the enzyme as a given amount of the enzyme may have a widely varying activity depending on reaction conditions.

It is important for developing a method to reduce the step of the measurement procedure. This invention describes a "mix and measure" method for assaying biological substance.

SUMMARY OF THE INVENTION

This present invention is for a method referred to as non-separation heterogenous assay that greatly simplifies the detection, identification , measurement of concentration and activity of biological substances. It is based on the change of the label signal due to the uneven distribution of the label between surface and liquid in a vessel after completion of the reaction among reactants. The method involves the coating of a reactant (labeled or unlabeled) onto a surface, addition of a sample with or without a competitor labeled using a label tag or unlabeled. The change of the label signal can be directly measured.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a new method for the detection, identification , measurement of concentration and activity of biological substances, which is based on the change of the label signal due to the uneven distribution of the label between surface and liquid in a vessel after completion of the reaction among reactants.

1. An assay in which the surface of a vessel is coated with reactant 1 which is a binding ligand for reactant 3 being the labeled form of reactant 2. Competitive binding reactions between reactant 2 and reactant 3 for reactant 1 or between reactant 2 and reactant 1 for reactant 3 are initiated when

a unknown amount of reactant 2 and a known amount of reactant 3 are added. The change of the label signal of the reactant 3 in the reaction vessel can be directly measured without an additional step and is directly proportional to the amount of reactant 2.

The reactions of the competitive binding between reactant 2 and reactant 3 for reactant 1 or between reactant 2 and reactant 1 for reactant 3 is interfered with by reactant 4 which is an inhibitor of reactant 2. The change of the label signal of the reactant 3 in the reaction vessel is directly proportional to the amount of reactant 4 (inhibitor).

2. An assay in which the surface of a vessel is coated with reactant 1 which is a labeled substrate for reactant 2 having a biological activity. The reaction is initiated when the reactant 2 is added. The change of the label signal in the reaction vessel can be directly measured and is proportional to the activity of reactant 2.

The reaction of hydrolyzation of reactant 1 coated on the surface of the vessel by reactant 2 is interfered with by reactant 3 which is an inhibitor of reactant 2. The change of the label signal is reciprocally proportional to the amount of reactant 3 (inhibitor).

EXAMPLES

The following examples are for an illustrative purpose only, and not to limit the scope of the invention.

Example 1

The detection of DNA hybridization

Materials: Single stranded deoxyribonucleic acid (ssDNA), fluorescence labeled complementary ssDNA (fluo-cDNA), sample double stranded DNA (dsDNA) and a microplate.

Method:

1. Immobilization of ssDNA: ssDNA is added into the wells of a microplate containing spacer and incubated for the covalent end-linkage of DNA.

2. DNA assay:

(1). A series of concentrations of dsDNA in a buffer (50 ul/well) and sample are added to the wells of the microplate immobilized with ssDNA.

(2). A fixed amount of the fluo-cDNA (50 ul/well) is added to each well containing dsDNA and the control.

(3). Competitive hybridization between the fluo-cDNA and the dsDNA with the immobilized ssDNA are initiated by incubating at 85^0 C for a while and cool to room temperature for 1 h.

(4). The change of the fluorescent intensity of the fluo-cDNA in the wells of the microplate is determined using a fluorometer and is directly proportional to the amount of dsDNA.

Example 2

Competitive assay for an antigen

Materials: An antigen, fluorescence labeled antibody, sample containing the antigen and a microplate.

Method:

1. Coating of antigen: The antigen (100ul/well) is added into the wells of a microplate and incubated at 37^0 C for 3 h. Then the microplate is washed 3 times using PBST.

2. Antigen assay:

(1). A series of concentrations of the antigen in a buffer (50 ul/well) are added to the wells of the coated microplate.

(2). A fixed amount of the fluo-antibody (50 ul/well) is added to each well containing the antigen and the control.

(3). Competitive binding reactions between the immobilized antigen and the free antigen (competitor) to the fluo-antibody are initialed by adding the fluo-antibody and incubated at 37° C for 2 h.

(4). The change of the fluorescent intensity of the fluo-antibody in the wells of the microplate is determined using a fluorometer and is directly proportional to the amount of the antigen in the sample.

Example 3

Fluorescent assay for protease and protease inhibitor

Materials: Protease, protease inhibitor, fluorescence labeled casein and a microplate.

Method:

1. Coating of a fluo-casein: fluo-casein (100ul/well) in PBS (pH 7.2) is added into the wells of a microplate and incubated at 37° C for 3 h and then the microplate is washed 3 times using PBST.

2. Protease activity assay:

A series of concentrations of a protease in a buffer (100 ul/well) and sample are added to the wells of the coated microplate and incubated at room temperature for 30 min. The change of the fluorescent intensity of the label in the wells of the microplate is measured with a fluorometer

and is directly proportional to the activity of the protease.

3. Protease inhibitor assay:

(1). Varying amounts of the protease inhibitor in the buffer (50 ul/well) and sample are added to the wells of the coated microplate. Negative and positive controls are included.

(2). A fixed concentration of the protease in the buffer (50 ul/well) is added to the wells containing inhibitor and the controls and incubated at room temperature for 1 h. The change of the fluorescent intensity of the label in the wells of the microplate is measured with a fluorometer and is reciprocally proportional to the amount of the inhibitor.

CLAIMS

I claim:

1. A competitive method for measuring the amount of a biological substance utilizing a competitive binding between the biological substances comprising:

a. the surface of a vessel coated with reactant 1;

b. a known amount of reactant 3 linked with a label and an unknown amount of reactant 2. The competitive reactions existing between reactant 2 and reactant 3 to bind to reactant 1 or between reactant 1 and reactant 2 to bind to reactant 3.

c. determining the change of the label signal of reactant 3 in the reaction vessel wherein the intensity of the label signal in the vessel is directly proportional to the amount of reactant 2.

2. The method of claim 1 wherein said reactant 1 is a receptor, said reactant 2 is a receptor binding ligand and said reactant 3 is the labeled form of reactant 2.

3. The method of claim 1 wherein said reactant 1 is a receptor binding ligand, reactant 2 is a

receptor and said reactant 3 is the labeled form of reactant 2.

4. The method of claim 1 wherein said reactant 1 is a lectin, said reactant 2 is lectin binding ligand and said reactant 3 is the labeled form of reactant 2.

5. The method of claim 1 wherein said reactant 1 is a lectin binding ligand, said reactant 2 is a lectin and said reactant 3 is the labeled form of reactant 2.

6. The method of claim 1 wherein said reactant 1 is an enzyme, said reactant 2 is an inhibitor and said reactant 3 is the labeled form of reactant 2.

7. The method of claim 1 wherein said reactant 1 is an inhibitor, said reactant 2 is an enzyme and said reactant 3 is the labeled form of reactant 2.

8. The method of claim 1 wherein said reactant 1 is an antigen, said reactant 2 is an antibody and said reactant 3 is the labeled form of reactant 2.

9. The method of claim 1 wherein said reactant 1 is a antibody, reactant 2 is an antigen and said reactant 3 is the labeled form of reactant 2.

10. The method of claim 1 wherein said reactant 1 is a single stranded DNA (ssDNA), said reactant 2 is DNA containing complementary sequence of ssDNA and said reactant 3 is the labeled complementary ssDNA.

11. The method of claim 1 wherein said label is selected from group of: fluorescent label, luminescent label, chromogenic label, and enzyme.

12. A method for detecting the biological activity of a biological substance utilizing the degradation of a substrate comprising:

a. the surface of a vessel coated with reactant 1 linked with a label.

b. addition of reactant 2 which has biological activity into the reaction vessel, said reactant

1 being hydrolyzed due to the activity of said reactant 2.

c. measuring the intensity of the label signal in the reaction vessel, wherein the change of the label signal in the vessel is directly proportional to the biological activity of said reactant 2.

13. The method of claim 12 wherein said reactant 2 is an enzyme and said reactant 1 is a substrate for the enzyme.

14. The method of claim 13 wherein said substrate is a polymeric or an oligomeric substrate.

15. The method of claim 13 wherein said enzyme is an enzyme that is able to cleave the substrate.

16. The method of claim 14 wherein said polymeric substrate is selected from group of: carbohydrate, DNA, RNA, protein, PEG, or polypeptide.

17. The method of claim 14 wherein said oligomeric substrate is selected from group of: peptide, oligosaccharide, or oligonucleotide.

18. The method of claim 13 wherein said enzyme is a protease or proteinase and said substrate is a protein.

19. The method of claim 13 wherein said enzyme is a carbohydrate hydrolase and said substrate is a carbohydrate.

20. The method of claim 13 wherein said enzyme is a DNase and said substrate is a DNA.

21. The method of claim 13 wherein said enzyme is a RNase and said substrate is a RNA.

22. The method of claim 13 wherein said enzyme is a peptidase and said substrate is a peptide.

23. The method of claim 13 wherein said enzyme is a oligosaccharide hydrolase and said substrate is a oligosaccharide.

24. The method of claim 12 wherein said label is selected from group of: fluorescent label, luminescent label, chromogenic label, and enzyme.

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